Investigation on the Toxic & Teratogenic Effects of GRAS Substances on the Developing Chick Embryo **Phossphated Mono & Di-glycerides** No Date

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Investigations on the Toxic and Teratogenic Effects of GRAS Substances on the Developing Chick Embryo.[1]

Phosphated Mono- and Di-glycerides

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[1]Report of investigations conducted under Contract No. 72-343 with the Food and Drug Administration, PHS, DHEW.

General Protocol:

Ten test substances were supplied by the Food and Drug Administration for testing in the chick embryo. Details on the nature and source of these substances is shown in Table 1. All substances were stored at room temperature in the dark until they were used, except that the propyl gallate and phosphated mono- and di-glycerides were kept under refrigeration. Most of the substances were dissolved in a suitable solvent or suspended in a suitable liquid for injection into fertile eggs. In one instance the substance was injected directly without a solvent or carrier. Specific information about solvents, solubility of the substances and problems peculiar to individual substances will be given under specific protocol for each substance tested.

Fertile eggs used in these investigations were from a specific pathogen free flock of Dekalb 161 egg production type chickens fed a breeder ration free of antibiotics or other drugs. Eggs were stored at 55° F and a relative humidity of 80 percent for 0 to 5 days prior to use. Eggs were allowed to reach room temperature, placed on plastic flats and subjected to ultraviolet irradiation for 30 minutes. The top of each egg was cleansed by a cotton swab saturated with 70 percent ethanol, a small hole was drilled over the air cell through the shell and the test substance was injected with the aid of a 0.25 ml. tuberculin syringe fitted with a suitable needle. All equipment and glassware used to handle the test substances or their solutions or suspensions were sterilized by auto claving and every attempt was made to avoid microbiological contamination of the eggs. Following injection the hole in each egg was sealed by a drop of flexible collodion and the eggs were set in or returned to the incubators. Jamesway Model 252 Incubator-Hatchers were used and maintained at 100° F dry bulb temperature and 86° F wet bulb temperature during the first [illegible] of incubation. Eggs were turned automatically each 4 hours. Eggs were candled periodically to remove dead embryos and all embryos were examined for stage of development and obvious defects. After 18 days of incubation viable embryos were transferred to hatching baskets and hatching temperature was reduced to 98.5° F dry bulb reading and humidity was increased to a 90° F wet bulb reading. Upon hatching (22nd day) chicks were examined for abnormalities and samples were cleared and alizarin stained to examine them for skeletal defects. Other embryos (50 for each substance studied) were sacrificed and samples of liver, muscle, bursa, brain, eye, spleen, heart, pancreas, lung and kidney were taken and fixed in formalin. Later tissues were embeded in paraffin, cut, stained and mounted for histopathological examination. Each sample was done in duplicate and hence a total of 10,000 tissues were examined for lesions.

Preliminary range finding experiments were conducted to find the doses of the test substances that could be used in constructing dose response curves for toxicity as measured by embryonic mortality. In two cases, the test substance was non-toxic in the largest dose that could be accommodated by injection. Specific dose response experiments using 100 or more eggs per dose and 5 or more doses of the test substance were conducted at a minimum of 3 time intervals to obtain the toxicity data reported. Solvent or sham injected controls and untreated control groups of eggs were used with each experiment. In some cases, extra trials were conducted to provide embryos for examination at critical doses of the test substances in order to further evaluate teratogenic response and obtain additional data on the nature of embryonic defects.

Data obtained from the experiments (except that from the range finding [illegible]) were transferred to data sheets provided (FDH form 2572, 2572a and [illegible]) and submitted to FDA for statistical analysis. Nine types of data summaries [illegible] 2 statistical treatments of the data were provided by FDA on the data submitted. The results presented and interpretations made are largely based on these data summaries.

Table i

FDA Project Test Substances

Test Substance and Identification		Compound No.
1. Lactose, Edible Fermont Dairies, Inc. Appleton, Wisc.		000063423
2. Propyl Gallate Lot 337		000121799
3. Sodium Ascorbate, U.S.P. FCC Lot No. 965102 Hoffmann-LaRoche Inc., Nutley, N. J. FDA 3167 73(C)		000134032
4. Sodium Erythorbate F.C.C. Lot No. 834072 FDA 3167 73(C) Hoffmann-LaRoche, Nutley, N. J.		977052064
5. Oil Nutmeg NF, East Indian Fritzsche Dodge & Olcott, Inc. 71-28 New York, N. Y.		MX 8008455
6. Zinc Sulfate - Rayon Lot # 2132R1 Virginia Chemicals, Inc. [illegible]	Anhyd. Monohyd.	007733020 007446197
7. Stannous Chloride, AR $2H_2O$ Mallinckrodt Chemical Works St. Louis, Mo.		007772998
8. Talc USP #141, Whittaker, Clark and Daniels, Inc.		010101390
9. Carob Bean Gum FDA 71-14		PM 9000402
10. Phosphated Mono- and Di-Glycerides Lot No. 126 Witco Chemical Organics Division New York, N. Y. EMCOL D70-30C		977051323

General Discussion and Comparisons:

A comparison of the relative toxicity of the ten compounds tested is shown in Table ii. When toxicity is evaluated by the air cell route of injection at 24 hrs. of incubation, which was the most sensitive for most of the substances tested, it may be seen that the test substances can be divided into 3 categories of toxicity. Substances highly toxic are zinc sulfate, propyl gallate and carob bean gum. Moderate toxicity was encountered with sodium ascorbate, sodium erythorbate, oil of nutmeg and stannous chloride. Those substances of low toxicity were lactose, talc and phosphated mono- and di-glyceride.

Most of the substances tested produced general embryo toxic response as ascites and/or edema except for lactose and talc at the doses tested. Some specific structural defects were noted and seemed to be related to certain substances as shown in Table ii.

Table ii

Comparison of Ten Substances Tested
for Toxicity and Teratology

Substance Tested	LC_{50} via air cell at 96 hrs.	Specific Abnormalities Noted
Lactose	very large	none
Propyl Gallate	13 mgs./kg.	Ascites, edema, celosomia.
Sodium Ascorbate	100 mgs./kg.	Ascites, edema, celosomia, liver histopathology, head defects.
Sodium Erythorbate	84 mgs./kg.	Ascites, liver histopathology.
Oil of Nutmeg	240 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Zinc Sulfate	4 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Stannous Chloride	120 mgs./kg.	Ascites, edema, celosomia.
Talc	>200 mgs./kg.	none
Carob Bean Gum	23 mgs./kg.	Anophthalmia, phocomelia, micromelia, torticollis, celosomia.
Phosphated Mono- and Di-Glycerides	>3000 mgs./kg.	Ascites, anophthalmia, brachygnathia.

X. PHOSPHATED MONO- AND DI-GLYCERIDES

Specific Protocol:

This substance was a very high viscosity liquid at room temperature. It was dissolved in chloroform-methanol (2:1) for yolk injection and the volume of the solution was varied to achieve different dose levels. For air cell administration, no solvent was used because the chloroform-methanol mixture was highly toxic via this route of injection and a large hypodermic needle (13 gauge) could be used to inject the substance in various amounts without a solvent carrier. No attempt was made to sterilize the solution or the substance due to limited stability. Nine dose levels of phosphated mono- and di-glycerides were tested at both 0 and 96 hrs. of incubation and via both air cell and yolk routes of administration.

Results:

The data for phosphated mono- and di-glycerides is presented in Tables 37-40. Percent mortality was increased significantly by air cell injection at most of the higher levels of administration. This effect was greater when the substance was given at 0 hr. in contrast to the effect observed at 96 hrs. Yolk injections resulted in high solvent mortality and no significant increase in percent mortality due to the test substance was noted except at the highest level when given at 0 hr. of incubation. It appeared that most levels of the test substance served to reduce the toxicity of the chloroform-methanol solvent because many significant reductions in mortality were noted at the lower doses of the phosphated mono- and di-glycerides. Compared to the low levels of the test substance, mortality tended to increase as more test substance was [illegible] percent abnormal chicks hatched was increased significantly by higher levels of test substance when given via the air cell. No increase was observed due to yolk injection. Only when given at 0 hr. via the air cell did high levels of phosphated mono- and di-glycerides significantly increase H-S-V-L abnormalities. The only general embryonic defect related to treatment was exencephaly which was seen frequently with air cell administration. An increase in defects of the beak and eye were noted in the H-S-V-L category at the high levels of air cell injection particularly at 0 hr. The specific findings were anophthalmia and brachygnathia.

Discussion:

In contrast to most substances tested, the phosphated mono- and di-glycerides were more toxic via air cell injection at 0 hr. than they were at 96 hrs. of incubation. Only with air cell administration was a clear cut relationship of dose to toxicity established. This was noted both in terms of percent mortality and as percent abnormal chicks hatched. No computer statistical analysis of the air cell data was provided by FDA but Chi Square values were hand calculated. Overall this substance was slightly toxic and the LC_{50} at 96 hrs. via the air cell was estimated to be >3000 mgs./kg. at 0 hr. via the air cell the LC_{50} was estimated at 1000 mgs./kg.

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Table 37

DATA SUMMARY

Phosphated KO Glycerides Without Solvent
via Air Cell at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	386	10.36	7.25	1.03
Solvent	None	109	16.51	8.25	1.83
400	20.0	30	46.66	10.00	0
800	40.0	30	70.00[1a]	6.66	6.66
1,000	50.0	79	32.91[1a]	7.59	1.26
1,200	60.0	28	57.14[1a]	25.00	0
[illegible]	[illegible]	[illegible]	[illegible][1]	7.69	5.12
[illegible]	[illegible]	[illegible]	80.00[1a]	16.66	6.66
[illegible]	[illegible]	110	75.45[1]	18.18[2a]	10.00[3]
[illegible]	125.0	[illegible]	77.63[1]	18.42[2a]	10.52[3]
3,000	150.0	79	73.41	15.18[2a]	6.32

1 Difference from control group is highly significant

1a Difference from control group is significant

2a Difference from control group is significant

3 Difference from control group is highly significant

4 No computer probit analysis available on regression

5 No computer probit analysis available on regression

Table 39

[illegible]

[illegible] Injected in Glycerine: Mixture Solvent
via Air Cell at [illegible] hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	386	10.36	7.25	1.03
Solvent	None	110	14.54	7.27	3.63
400.0	20.0	30	13.33	3.33	0
800.0	40.0	30	20.00	10.00	3.33
1,000.0	50.0	79	25.31[1a]	10.12	5.06[3]
1,200.0	60.0	30	20.00	13.33	0
1,500.0	75.0	80	31.25[1a]	15.00	1.25
1,600.0	80.0	30	43.33[1a]	3.33	0
2,000.0	100.0	109	38.53[1]	19.26[2a]	4.58
2,500.0	125.0	79	34.17[1a]	15.18	0
3,000.0	150.0	80	37.50[1]	22.50[2a]	2.50

1 Difference from control group is highly significant

1a Difference from control group is significant

2a Difference from control group is significant

3 [illegible]

4 No computer probit analysis available on regression

5 No computer probit analysis available on regression

Table 39

DATA SUMMARY

Phosphated Mono Glycerides in Chloroform - Methanol via Yolk at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	386	10.36	7.25	1.03
Solvent	None	108	55.55	13.88	5.55
320.0	16.0	30	36.66	3.33	0
640.0	32.0	29	27.58[1a]	3.44	0
800.0	40.0	79	41.77	7.59	3.79
960.0	48.0	30	23.33[1]	0	0
1,200.0	60.0	80	45.00	2.50[2a]	0
1,280.0	64.0	29	44.82	24.13	3.44
1,600.0	80.0	109	42.20	4.58[2a]	0.91
2,000.0	100.0	30	57.5	12.50	3.75[3]
2,400.0	120.0	80	71.25[1a]	8.75	1.25

[1] Difference from control group is highly significant

[1a] Difference from control group is significant

[2a] Difference from control group response is significant

[3] NS

[4] E outside Range - too few points on curve

[5] NS - F (Cal.) $\leq$ F (.05)

Table 40

DATA SUMMARY

Phosphated MG Glycerides in Chloroform - Methanol via Yolk at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	386	10.36	7.25	1.03
Solvent	None	109	36.69	9.17	0
320.0	16.0	29	10.34[1a]	0	0
640.0	32.0	30	3.33[1]	6.66	0
800.0	40.0	80	12.50[1]	6.25	1.25
960.0	48.0	30	13.33[1a]	0	0
1,200.0	60.0	77	11.68[1]	3.89	0
1,280.0	64.0	30	16.66	23.33[2]	0
1,600.0	80.0	109	17.43[1]	18.34	0.91
2,000.0	100.0	80	21.25[1a]	16.25	0
2,400.0	120.0	79	22.78	18.98	2.53[3]

[1] Difference from test control group is highly significant

[1a] Difference from test control group is significant

[2] NS

[3] NS

[4] NS - F (Cal) < F (.05)

[5] Slope is negative

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Zinc Sulfate	4 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Stannous Chloride	120 mgs./kg.	Ascites, edema, celosomia.
Talc	>200 mgs./kg.	none
Carob Bean Gum	23 mgs./kg.	Anophthalmia, phocomelia, micromelia, torticollis, celosomia.
Phosphated Mono- and Di-Glycerides	>3000 mgs./kg.	Ascites, anophthalmia, brachygnathia.